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The Alcohol Dehydrogenase Gene *adhA* in *Corynebacterium glutamicum* Is Subject to Carbon Catabolite Repression▽

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Corynebacterium glutamicum **has recently been shown to grow on ethanol as a carbon and energy source and to possess high alcohol dehydrogenase (ADH) activity when growing on this substrate and low ADH activity when growing on ethanol plus glucose or glucose alone. Here we identify the *C. glutamicum* ADH gene (*adhA*), analyze its transcriptional organization, and investigate the relevance of the transcriptional regulators of acetate metabolism RamA and RamB for *adhA* expression. Sequence analysis of *adhA* predicts a polypeptide of 345 amino acids showing up to 57% identity with zinc-dependent ADH enzymes of group I. Inactivation of the chromosomal *adhA* gene led to the inability to grow on ethanol and to the absence of ADH activity, indicating that only a single ethanol-oxidizing ADH enzyme is present in *C. glutamicum*. Transcriptional analysis revealed that the *C. glutamicum adhA* gene is monocistronic and that its expression is repressed in the presence of glucose and of acetate in the growth medium, i.e., that *adhA* expression is subject to catabolite repression. Further analyses revealed that RamA and RamB directly bind to the *adhA* promoter region, that RamA is essential for the expression of *adhA*, and that RamB exerts a negative control on *adhA* expression in the presence of glucose or acetate in the growth medium. However, since the glucose- and acetate-dependent down-regulation of *adhA* expression was only partially released in a RamB-deficient mutant, there might be an additional regulator involved in the catabolite repression of *adhA*.**

Corynebacterium glutamicum is a gram-positive, aerobic soil bacterium that is widely used in the biotechnological production of amino acids such as L-glutamate and L-lysine (30, 36). The organism grows on a variety of carbohydrates and organic acids as single or combined sources of carbon and energy, and we recently also showed the growth of *C. glutamicum* on ethanol (3). This substrate is oxidized via acetaldehyde to acetate, which enters the tricarboxylic acid cycle after activation to acetyl coenzyme A (acetyl-CoA). As in the case of the growth of *C. glutamicum* with acetate as the sole carbon source, the glyoxylate cycle plays an essential role in the anaplerotic function during growth on ethanol (3). Whereas the acetate-activating enzymes acetate kinase (AK) and phosphotransacetylase (PTA) and, also, the key enzymes of the glyoxylate cycle, isocitrate lyase (ICL) and malate synthase (MS) and their respective genes (*ack*, *pta*, *aceA*, and *aceB*) have been intensively investigated (reviewed in reference 20), much less is known about the alcohol and acetaldehyde dehydrogenases (ADH and ALDH, respectively), which are involved in ethanol's oxidation to acetate. And although the genome sequence of *C. glutamicum* has been determined and annotated (26, 29), the genes encoding these two enzymes have not been identified and a functional characterization has not yet been performed.

In contrast to the growth of *C. glutamicum* on many substrate mixtures, such as glucose plus acetate, lactate, pyruvate, or fructose (7, 12, 52), the growth of this organism on a mixture of glucose and ethanol is biphasic, with glucose consumption in the first and ethanol consumption in the second exponential growth phase (3). This biphasic growth behavior is probably due to relatively low ADH and ALDH activities in the first and much higher ADH and ALDH activities (and thus, high ethanol oxidation activity) in the second growth phase (3). Diauxic growth of *C. glutamicum* and the sequential utilization of carbon sources have been reported so far only for its growth in medium containing glucose and glutamate, and here the biphasic growth behavior is due to the repression of the glutamate uptake system in the presence of glucose (32, 33). Interestingly, so far there is no evidence for a global carbon catabolite repression system in *C. glutamicum*, such as the CcpA-dependent carbon control system in *Bacillus subtilis* and other low-GC, gram-positive bacteria (38, 51) or the cyclic AMP (cAMP)-dependent cAMP receptor protein system in *Escherichia coli* (6, 45). However, Letek et al. (35) recently reported on the sugar-mediated repression of genes (*gntP* and *gntK*) involved in gluconate metabolization in *C. glutamicum* and showed that GlxR, a cAMP-dependent transcriptional regulator, is involved in this regulation mechanism. However, biphasic growth or the sequential utilization of glucose and gluconate has not been shown for the growth of *C. glutamicum* in medium containing glucose plus gluconate, and thus, the significance of GlxR for a sequential utilization of two or more carbon sources remains to be investigated.

Recently, we identified two novel regulatory proteins, designated as *r*egulators of *a*cetate *m*etabolism *A* and *B*, i.e., RamA and RamB (10, 19). Both proteins were shown to be transcriptional regulators of the AK, PTA, ICL, and MS genes, RamA being an activator in the presence of acetate and RamB being a repressor when *C. glutamicum* grows on glucose as the sole carbon and energy source. Both regulators are subject to negative autoregulation, and additionally, *ramB* expression is subject to carbon source-dependent positive control by RamA

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TABLE 1. Strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristic(s) or sequence	Source, reference, or purpose
Strains		
E. coli DH5α	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	25
E. coli BL21(DE3)	*ompT hsdS$_B$* ($r_B^-m_B^-$) *gal dcm* (DE3)	44
WT *C. glutamicum*	WT strain ATCC 13032	American Type Culture Collection
C. glutamicum AA1	WT strain with integration of pDrive in *adhA*	This work
C. glutamicum RG1	WT strain with deletion of *ramB*	19
C. glutamicum RG2	WT strain with deletion of *ramA*	10
Plasmids		
pDrive	Cloning vector carrying the *lacZ* gene, Km^r Amp^r	QIAGEN, Hilden, Germany
pDrive-*adhA*_int	pDrive containing an internal 509-bp fragment of the *adhA* gene	This work
pET2	Promoter probe vector carrying the promoterless *cat* gene, Km^r	49
pET2-*adhAp$_1$*	pET2 containing a 401-bp insertion of the *adhA* promoter region	This work
pET2-*adhAp$_2$*	pET2 containing a 236-bp insertion of the *adhA* promoter region	This work
pET2-*adhAp$_3$*	pET2 containing a 213-bp insertion of the *adhA* promoter region	This work
pET28-RamAx6His	pET28 containing the *ramA* gene	10
pET29-RamBx6His	pET29 containing the *ramB* gene	19
Oligonucleotides		
adhA-hin	5′-AAACTGCAGAACCGGCAGGGAAACTCAGT-3′	Primer for *adhA* integration PstI site (underlined)
adhA-rev	5′-CGGGATCCCGGGGAACTCTCCCGGTGG-3′	Primer for *adhA* integration and Southern hybridization probe, BamHI site (underlined)
adhA-SB-hin	5′-GCGTCGACGTCTGCCAGAAGAGCAATTTGCCA-3′	Forward primer for Southern and Northern hybridization probes, SalI site (underlined)
adhA-NB-rev	5′-CTAGTCTAGACTAGCCAAACCGCTCTGCAGCAACC-3′	Backward primer for Northern hybridization probe, XbaI site (underlined)
adhA-P2-hin	5′-GCGTCGACGTCTCCAGTTCATTCACGGTTGTT-3′	Primer for amplification of the 236-bp *adhAp$_2$* promoter fragment, SalI site (underlined)
adhA-P1-hin	5′-GCGTCGACGTGGCTGGGGGCATCGAA-3′	Primer for amplification of the 401-bp *adhAp$_1$* promoter fragment, SalI site (underlined)
adhA-P2-rev	5′-CGCGGATCCGCGGGTGCAGCAGTGGTCATAA-3′	Backward primer for *adhA* promoter fragments *adhAp$_1$* and *adhAp$_2$*, BamHI site (underlined)
adhA-P3-rev	5′-GCGGATCCAAGTTGAGTTATTACCCCCGAACA-3′	Backward primer for *adhA* promoter fragment *adhAp$_3$*, BamHI site (underlined)
CM4	5′-GAAAATCTCGTCGAAGCTCG-3′	50
CM5	5′-AAGCTCGGCGGATTTGTC-3′	43

(8, 9). RamA binds to single or tandem stretches of A/C/TG$_{4-6}$T/C or AC$_{4-5}$A/G/T, whereas RamB specifically binds to a highly conserved 13-bp motif (AA/GAACTTTGCAAA). Due to their regulatory function as transcriptional regulators of the AK, PTA, ICL, and MS genes and since all these enzymes are also involved in ethanol metabolism, both RamA and RamB are likely candidates to also be involved in the regulatory control of the genes encoding the ethanol-oxidizing enzymes.

In the present study, we identified the ADH gene (*adhA*), which is involved in the growth of *C. glutamicum* on ethanol. Furthermore, we analyzed the transcription of *adhA* and provide evidence for carbon source-dependent transcriptional regulation of this gene. Finally, we investigated the regulatory functions of RamA and RamB in *adhA* expression control.

MATERIALS AND METHODS

Bacteria, plasmids, oligonucleotides, and culture conditions. Bacterial strains, plasmids and oligonucleotides, as well as their relevant characteristics and sources, are given in Table 1. TY medium (42) was used as complex medium for *C. glutamicum* and *Escherichia coli*. The minimal medium used for *C. glutamicum* has been described previously (14) and contained ethanol, glucose, or acetate or a mixture of those carbon sources in the concentrations indicated in Results. When appropriate, the medium contained kanamycin (50 μg ml^{-1} for strains carrying plasmids and 15 μg ml^{-1} for integration mutants and for transformant selection). *C. glutamicum* was grown aerobically at 30°C and *E. coli* at 37°C in 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. The growth of the bacteria was followed by measuring the optical density at 600 nm.

DNA preparation and transformation. The isolation of chromosomal and plasmid DNA from *C. glutamicum* was performed as described previously (14), and plasmid isolation from *E. coli* was carried out according to the method of Birnboim (5). DNA transfer into *C. glutamicum* was performed by electroporation, and recombinant strains were selected on LBHIS agar plates (48). Transformation of *E. coli* was carried out according to the method of Inoue et al. (27).

PCR techniques. The PCR experiments were performed in a Biometra personal cycler (Biotron) with *Taq* polymerase (MBI-Fermentas). The oligonucleotides were obtained from MWG-Biotech or from biomers.net GmbH. The cycling times and temperatures were chosen according to fragment length and primer constitution. The PCR products were separated on agarose gels and purified using a Nucleospin extract kit (Macherey Nagel).

TABLE 2. Specific ADH activities of different *C. glutamicum* strains

Strain	Specific ADH activity (U/mg protein)[a]				
	EtOH	Glc	EtOH/Glc[b]	Ac	EtOH/Ac[b]
C. glutamicum WT	1.85 ± 0.24	0.22 ± 0.09	0.36 ± 0.05	0.06 ± 0.03	0.09 ± 0.05
C. glutamicum AA1 (*adhA*-negative)	NG	<0.01	<0.01	ND	ND
C. glutamicum RG1 (*ramB*-negative)	2.07 ± 0.31	0.67 ± 0.14	0.65 ± 0.08	0.31 ± 0.01	0.41 ± 0.01
C. glutamicum RG2 (*ramA*-negative)	NG	<0.01	<0.01	NG	NG

[a] The strains were grown in minimal medium containing 1% ethanol (EtOH), 1% glucose (Glc), Glc plus EtOH (1% each), 1% acetate (Ac), or Ac plus EtOH (1% each). The values are the means ± standard deviations of the results of three independent experiments. NG, not grown; ND, not determined.
[b] Cells were harvested in the first exponential phase of growth.

DNA manipulation and Southern hybridization. Restriction enzymes, T4 DNA ligase, calf intestinal phosphatase, RNase A, and proteinase K were obtained from MBI-Fermentas and used as instructed by the manufacturer. DNA hybridization experiments were performed as described previously (14). An *adhA*-specific 880-bp DNA fragment was amplified from the chromosomal DNA of wild-type (WT) *C. glutamicum*, labeled with digoxigenin-dUTP by PCR with primers *adhA*-SB-hin and *adhA*-rev, and used as a probe. The labeling, hybridization, washing, and detection were performed by using a nonradioactive DNA labeling and detection kit from Roche Diagnostics, following the manufacturer's instructions.

Construction of a *C. glutamicum adhA* integration mutant. The inactivation of the chromosomal *adhA* gene in *C. glutamicum* was performed by integration of the vector pDrive into the *adhA* gene. An internal fragment covering the region between base pair 321 of the 5′ end and base pair 208 of the 3′ end of the *adhA* gene was generated by using primers *adhA*-hin and *adhA*-rev and ligated via the A/T overhangs into pDrive, and the ligation mixture was transformed into *E. coli*. The identification of positive *E. coli* clones was performed by blue/white screening on TY agar plates containing kanamycin, isopropyl-β-D-thiogalactopyranoside (IPTG; 50 μM), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 80 μg/ml). Recombinant plasmids were isolated from *E. coli* and electroporated into WT *C. glutamicum*. The integration of pDrive into the chromosomal *adhA* locus of the resulting strain, *C. glutamicum* AA1, was verified by Southern blot analysis. For this purpose, a labeled *adhA* probe was hybridized to HindIII-restricted and size-fractioned chromosomal DNA from WT *C. glutamicum* and *C. glutamicum* AA1. The hybridization resulted in one signal of about 10 kb with DNA from the WT strain and in two signals of about 11 kb and 3.3 kb with DNA from the mutant. According to the restriction map of the *adhA* locus, these sizes were expected.

Cloning of *adhA* promoter fragments. The $adhAp_1$ (401 bp), $adhAp_2$ (236 bp), and $adhAp_3$ (213 bp) promoter fragments were amplified from the chromosomal DNA of WT *C. glutamicum*. The PCR products were digested with SalI and BamHI, ligated into the SalI/BamHI-restricted plasmid pET2, and transformed into *E. coli*. The recombinant plasmids pET2-$adhAp_1$, pET2-$adhAp_2$, and pET2-$adhAp_3$ were isolated from *E. coli* and introduced into *C. glutamicum*.

RNA techniques. For RNA isolation, *C. glutamicum* cells were grown in minimal medium containing glucose or ethanol, harvested in the exponential growth phase (optical density at 600 nm of about 4.5), and treated with 1 volume of ice-cold killing buffer (20 mM Tris-HCl, pH 8.0, 20 mM NaN_3, 5 mM $MgCl_2$). The isolation procedure was performed as described previously (43), and aliquots of the RNA were stored at −70°C until use.

For Northern (RNA) hybridization, an 1,149-bp DNA fragment was amplified from the chromosomal DNA of WT *C. glutamicum* by PCR with primers *adhA*-SB-hin and *adhA*-NB-rev, digested with SalI and XbaI, purified with a Nucleospin extract kit, and used as a probe. The [α-^{32}P]dATP labeling of the probe was performed with Klenow fragment (MBI-Fermentas). The separation of unincorporated nucleotides was done by using MicroSpin G-25 columns (Amersham Biosciences). For the hybridization, about 10 μg of total RNA from WT *C. glutamicum* was separated on an agarose gel containing 17% (vol/vol) formaldehyde and transferred onto a nylon membrane (14). Filter hybridization and detection were accomplished with conventional protocols (42).

The primer extension reactions were carried out as described previously (43) with IRD800-labeled primers CM4 and CM5 and RNA from ethanol-grown *C. glutamicum*(pET2-$adhAp_2$) cells, and the primer extension products were analyzed with an automatic sequencer (LI-COR 4000L; Licor, Inc.) using a 6% (wt/vol) polyacrylamide gel at 1,500 V and 50°C. For the exact localization of the transcriptional start site, sequencing reactions using plasmid pET2-$adhAp_2$ and the same oligonucleotide used for the respective primer extension reaction were coelectrophoresed in the sequencing reaction mixture.

Promoter binding assays with His-tagged RamA and RamB fusion protein. The construction of plasmids for the preparation of His-tagged RamA and RamB and the synthesis and purification of these proteins have been described previously (10, 19). The binding of purified RamA or RamB to the *adhA* promoter region was tested by DNA electrophoretic mobility shift assays (EMSAs) using the fragments $adhAp_1$, $adhAp_2$, and $adhAp_3$. The EMSA experiments were carried out as described previously (10).

Enzyme assays. For the determination of enzyme activities in cell extracts, *C. glutamicum* cells were grown in minimal medium containing the respective carbon source(s) and harvested in the exponential growth phase. Cell extracts were prepared as described previously (3).

ADH activity was analyzed in the acetaldehyde-forming direction as described previously (3). Chloramphenicol acetyltransferase (CAT) activity was determined as described by Schreiner et al. (43).

Computational analysis. For the analysis of the $\Delta G^{\circ\prime}$ value (free energy under standard conditions) of the *adhA* terminator structures, the program Clone Manager 7 (Sci Ed Central) was used. Databank searches and alignments were carried out using BLAST, CLUSTAL W, and BioEdit software (1, 24, 47).

RESULTS

Identification and characterization of the *adhA* gene. We previously found relatively high specific activities of ADH in ethanol-grown cells of *C. glutamicum* (3). Five genes (*cg0273*, *cg0387*, *cg0400*, *cg2714*, and *cg3107*) were annotated as potential ADH genes in the genome of *C. glutamicum* (GenBank accession numbers NC_003450 and NC_006958) (26, 29). Amino acid sequence alignments of the deduced polypeptides with ADH enzymes from organisms known to utilize ethanol indicated Cg3107 to be the most likely candidate as ADH involved in the ethanol oxidation of *C. glutamicum*. Cg3107 showed 47% identity to the functionally analyzed ADHs of *Pichia stipidis* and *Saccharomyces cerevisiae*, 49% identity to the thermostable ADH of the gram-positive *Bacillus stearothermophilus*, and 57% identity to the NAD^+-dependent ADH of the gram-negative *Pseudomonas aeruginosa*. The other four proteins (Cg0273, Cg0387, Cg0400, and Cg2714) showed less similarity (<40%) to all these ADHs. Therefore, *cg3107* was inactivated and the resulting mutant *C. glutamicum* AA1 was analyzed for growth on ethanol. In fact, this mutant was not able to grow in minimal medium containing ethanol as the single carbon source, whereas its growth on glucose or acetate was not affected (data not shown). For confirmation, the specific ADH activities in cell extracts of WT *C. glutamicum* and AA1 were determined. In contrast to the WT strain, the mutant did not show any ADH activity on any substrate tested (Table 2). This result and the inability of the mutant to grow on ethanol indicate that *cg3107* in fact represents the ADH gene, and therefore, we designated it as *adhA*.

The *adhA* gene of *C. glutamicum* consists of 1,038 bp and is preceded by a typical ribosomal binding site (AGAAGG). A

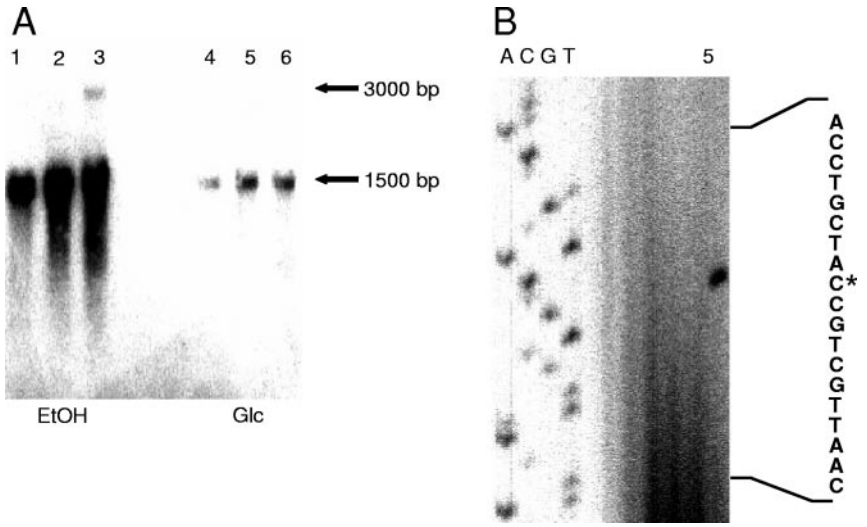

FIG. 1. Transcriptional characterization of the *adhA* gene. (A) Northern blot analysis after growth of *C. glutamicum* in minimal medium containing ethanol (lanes 1 to 3) or glucose (lanes 4 to 6) as carbon sources. Total RNA (5 μg in lanes 1 and 4; 10 μg in lanes 2 and 5; 15 μg in lanes 3 and 6) was electrophoresed and probed with a radioactively labeled *adhA*-specific DNA probe. Sizes are shown on the right. (B) Primer extension analysis of the transcriptional start site in front of the *adhA* gene. The primer extension product is shown in lane 5. Lanes A, C, G, and T represent the products of sequencing reactions with the same primer (CM4) used for the primer extension reaction. The relevant DNA sequence (coding strand) is shown on the right, and the transcriptional start site is indicated by an asterisk.

region of dyad symmetry is located 193 bp downstream of the *adhA* stop codon, and the mRNA hairpin loop predicted from this sequence has a $\Delta G^{\circ\prime}$ value of −21.5 kcal/mol at 25°C. This structure indicates transcriptional termination downstream of *adhA*. According to the nucleotide sequence, *adhA* encodes a polypeptide of 345 amino acid residues with a predicted molecular mass of 36.8 kDa. Upstream and downstream of *adhA*, there are genes encoding hypothetical or putative proteins of unknown functions.

Amino acid sequence alignments and BLAST analysis with the *C. glutamicum* ADH revealed homologous proteins in *Corynebacterium efficiens* YS-314 (GenBank accession no. CE0053) and *Corynebacterium diphtheriae* NCTC 13129 (GenBank accession no. DIP2114), showing identities of 72% and 74%, respectively, whereas no significant similarities to proteins of *Corynebacterium jeikeium* K411 were detected. Further comparisons of the *C. glutamicum* enzyme with *E. coli* proteins revealed no significant similarity to the bifunctional AdhE, which physiologically catalyzes ethanol formation from acetyl-CoA (22).

Transcriptional analysis of the *adhA* gene. To determine the size of the *adhA* transcript and to test whether the different specific ADH activities observed in *C. glutamicum* cells grown on either glucose or ethanol are due to transcriptional control, Northern blot hybridization experiments were performed. As shown in Fig. 1A, the hybridizations revealed their main signals at about 1.5 kb. With RNA from ethanol-grown cells, the signal was much more intense than that obtained with RNA from glucose-grown cells (Fig. 1A). These results indicate that the *C. glutamicum adhA* gene is monocistronic and that it is subject to carbon source-dependent transcriptional regulation.

To further investigate the carbon source-dependent transcription of the *adhA* gene, the three promoter fragments $adhAp_1$, $adhAp_2$, and $adhAp_3$ (Fig. 2A) were tested for activity. For this purpose, the plasmids pET2-$adhAp_1$, pET2-$adhAp_2$, and pET2-$adhAp_3$ were transformed into WT *C. glutamicum*, and the specific CAT activities in the resulting strains were determined during growth in minimal medium containing ethanol, glucose, acetate, and the substrate mixtures ethanol plus glucose or ethanol plus acetate. As shown in Table 3, WT *C. glutamicum*(pET2-$adhAp_1$) showed specific CAT activities of 6.89 U/mg protein on ethanol and about fourfold lower activities on glucose or ethanol plus glucose. Interestingly, on acetate or acetate plus ethanol, the specific CAT activities were even about 14-fold lower than on ethanol. In WT *C. glutamicum*(pET2-$adhAp_2$) containing the shortened *adhA* promoter fragment, the specific CAT activities on all substrates were five- to ninefold lower than in WT *C. glutamicum*(pET2-$adhAp_1$). WT *C. glutamicum*(pET2-$adhAp_3$) showed no CAT activity at all. These results indicate (i) that the *adhA* promoter resides in the a*dhA* upstream region covered by fragment $adhAp_2$ (position −218 to +1 with respect to the translational start codon), (ii) that the *adhA* promoter drives the carbon source-dependent expression of *adhA*, (iii) that fragment $adhAp_3$ does not contain a functional promoter, and (iv) that the region covered by fragment $adhAp_3$ contains an activating element leading in a carbon source-independent manner to an increased *adhA* promoter activity.

To investigate the carbon source-dependent expression of *adhA* in more detail, we performed successive growth experiments with *C. glutamicum*(pET2-$adhAp_2$) and *C. glutamicum*-(pET2-$adhAp_1$) in minimal medium containing glucose (1%), a glucose-ethanol substrate mixture (0.125% and 1%, respectively), ethanol (1%), and glucose again (1%) and determined the CAT activities (i.e., *adhA* promoter activities) in the course of the cultivations. Representative results for *C. glutamicum*-(pET-$adhAp_2$) are shown in Fig. 3. During growth on glucose, the specific CAT activity was relatively low (Fig. 3A). In the subsequent culture containing glucose plus ethanol, the growth was biphasic; CAT activity was low in the glucose-metabolizing

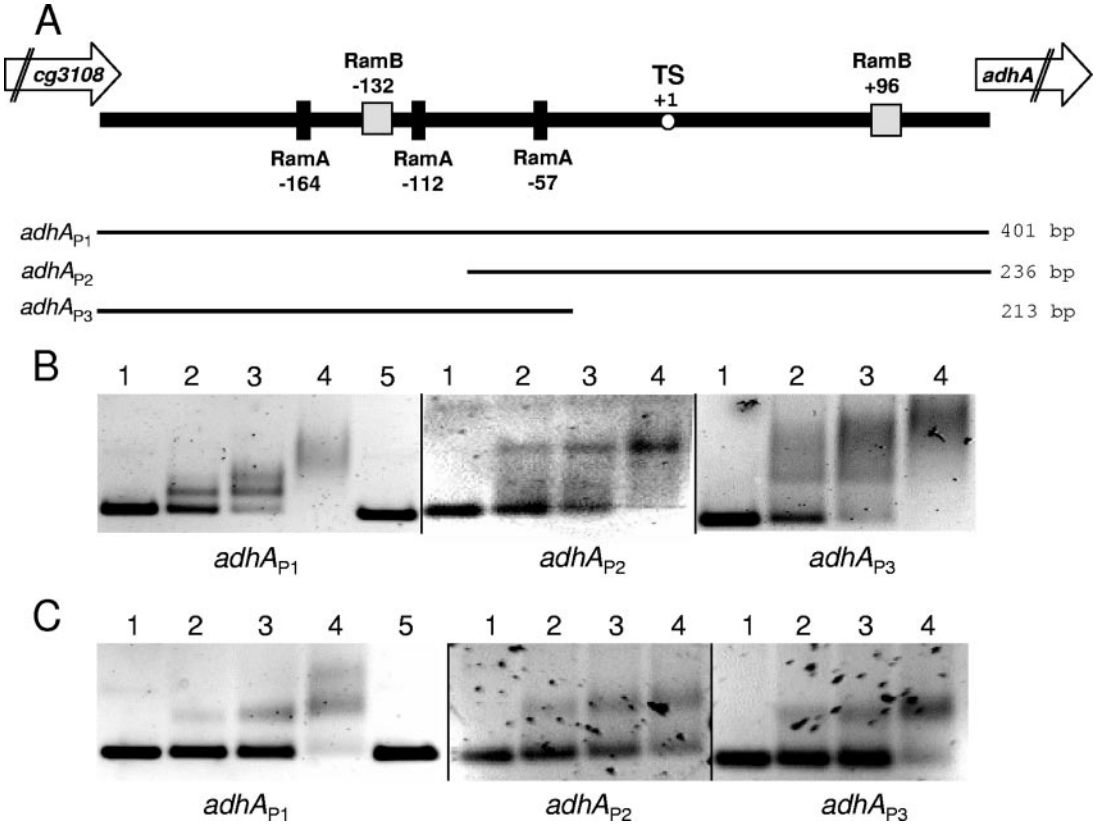

FIG. 2. Genomic locus of the *adhA* promoter region and DNA fragments used for mapping the relevant RamA and RamB binding sites (A) and representative EMSAs using hexahistidyl-tagged RamA (B) and RamB (C) proteins. cg3108 codes for a putatively secreted, unknown protein. The transcriptional start site is denoted as TS. The black boxes represent the putative RamA binding sites and the grey boxes the RamB binding sites. Their respective localizations relative to the transcriptional start site are given. The fragments used for the binding assays are given as bars and designated as indicated to the left, whereas the respective sizes are indicated to the right. The fragments used in the EMSAs with hexahistidyl-tagged RamA (B) and RamB (C) are indicated below the different parts of the gels. Lanes 1 to 4 show EMSAs using 0, 0.25, 0.5, and 1 μg of RamA or RamB, respectively. Lane 5 shows a negative control using 1 μg of bovine serum albumin protein with the respective probe.

growth phase and increased in the ethanol-metabolizing growth phase to a maximum of about 1 U/mg protein (Fig. 3B). When the strain was then grown on ethanol as the single carbon source, CAT activity was high in the exponential growth and slightly lower in the stationary phase (Fig. 3C). The following cultivation on glucose led to a decrease of the specific CAT activity to the level observed in the first cultivation with glucose as the sole substrate (Fig. 3D). The CAT activities determined during the cultivations with *C. glutamicum*(pET-*adhAp*$_1$) correlated well with those of *C. glutamicum*(pET-*adhAp*$_2$) except that they were five- to ninefold higher under all conditions. The CAT activities observed in all these cultivations also correlate well with the ADH activities of WT *C. glutamicum* previously observed in identical successive growth experiments (3). Taken together, the results corroborate that the carbon source-dependent regulation of ADH in *C. glutami-*

TABLE 3. Specific CAT activities of *C. glutamicum* strains carrying different *adhA* promoter fragments in plasmid pET2

Strain	Specific CAT activity (U/mg protein)[a]				
	EtOH	Glc	EtOH/Glc[b]	Ac	EtOH/Ac[b]
WT *C. glutamicum*(pET2-*adhAp*$_1$)	6.89 ± 0.88	1.57 ± 0.02	1.68 ± 0.04	0.43 ± 0.03	0.61 ± 0.03
WT *C. glutamicum*(pET2-*adhAp*$_2$)	1.33 ± 0.08	0.31 ± 0.01	0.18 ± 0.09	0.09 ± 0.03	0.15 ± 0.02
WT *C. glutamicum*(pET2-*adhAp*$_3$)	<0.01	<0.01	<0.01	ND	ND
C. glutamicum RG1(pET2-*adhAp*$_1$) (*ramB*-negative)	7.18 ± 0.04	3.37 ± 0.11	3.35 ± 0.02	1.39 ± 0.03	1.65 ± 0.03
C. glutamicum RG1(pET2-*adhAp*$_2$) (*ramB*-negative)	0.83 ± 0.06	0.24 ± 0.02	0.26 ± 0.01	0.07 ± 0.01	0.09 ± 0.01
C. glutamicum RG2(pET2-*adhAp*$_1$) (*ramA*-negative)	NG	0.01	0.01	NG	NG
C. glutamicum RG2(pET2-*adhAp*$_2$) (*ramA*-negative)	NG	<0.01	<0.01	NG	NG

[a] The strains were cultured in minimal medium containing 1% ethanol (EtOH), 1% glucose (Glc), Glc plus EtOH (1% each), 1% acetate (Ac), or Ac plus EtOH (1% each). The values are means ± standard deviations of the results of three independent experiments. ND, not determined; NG, not grown.

[b] In medium containing ethanol plus glucose and ethanol plus acetate, the cells showed biphasic growth behavior with preferential utilization of glucose or acetate, respectively. Cells were harvested in the first exponential phase of growth.

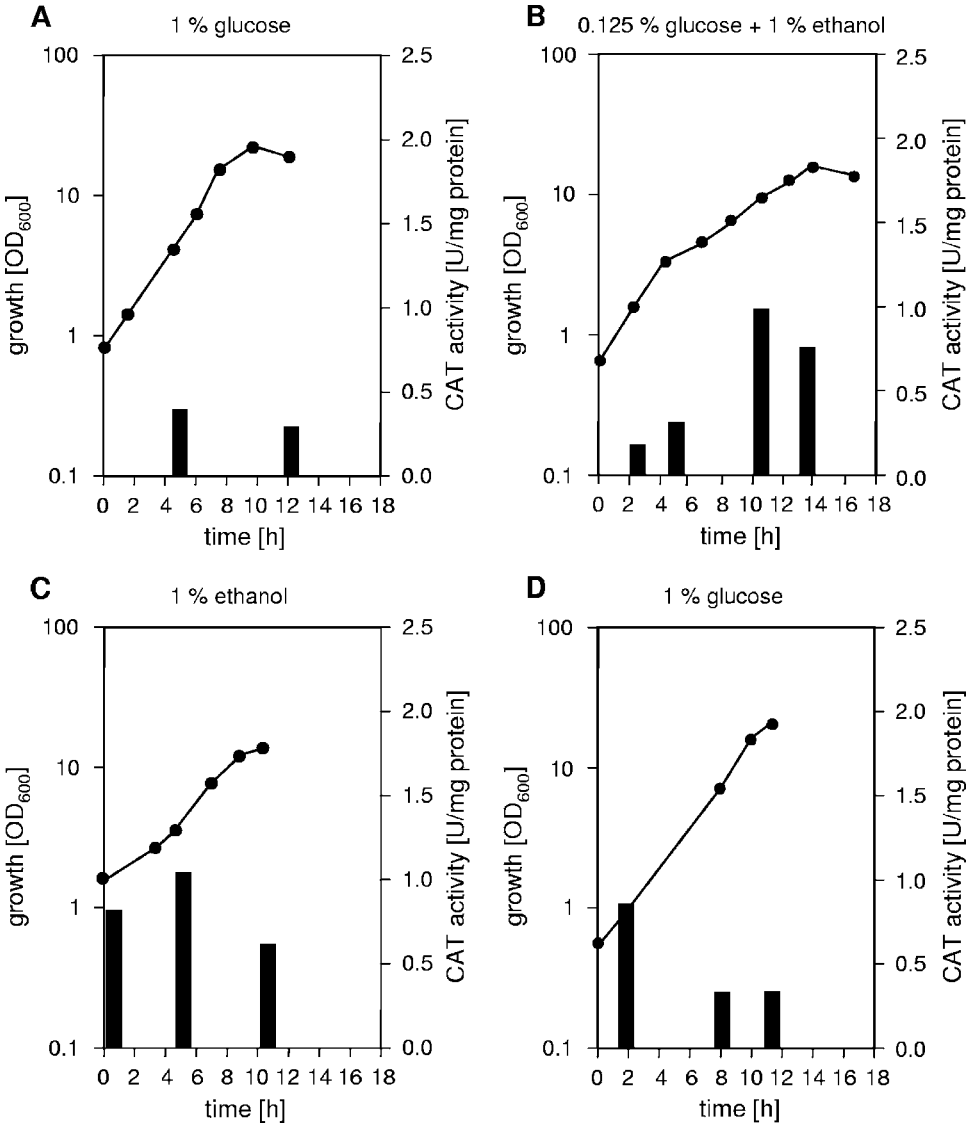

FIG. 3. *adhA* promoter activity during growth of WT *C. glutamicum*(pET2-*adhAP2*) on 1% glucose (A), 0.125% glucose and 1% ethanol (B), 1% ethanol (C), and 1% glucose (D). Black circles, growth; black bars, specific CAT activity.

cum and, also, the growth phase-dependent regulation of ADH on ethanol as the sole substrate take place at the transcriptional level.

To precisely localize the transcriptional initiation site of the *C. glutamicum adhA* gene, primer extension experiments were performed. With the use of oligonucleotide CM4 and 50 μg total RNA from *C. glutamicum*(pET2-*adhAp*$_2$), a signal corresponding to a G residue 130 nucleotides upstream of the *adhA* translational start codon ATG was observed (Fig. 1B). This result was confirmed in an independent experiment with a different primer (CM5) (data not shown). Centered 10 bp upstream of the *adhA* transcriptional initiation site, the sequence TAGGGT was found and designated as the −10 region. However, there is only relatively weak similarity to the consensus −10 sequence (TAT/CAAT) of corynebacterial promoters (39, 40). No apparent −35 region could be recognized, which is a common feature of *C. glutamicum* promoters (39, 40).

RamA and RamB bind to the *adhA* promoter region. AK, PTA, ICL, and MS are essential enzymes for acetate metabolism in *C. glutamicum* and are also required for the growth of *C. glutamicum* on ethanol (3). The expression of the genes encoding these four enzymes has been shown to be transcriptionally controlled by RamA and RamB (see the introduction). Interestingly, we observed three potential RamA binding sites in the promoter region of *adhA*, i.e., AGGGGGT, located 164 bp upstream of the transcriptional start, and CGGGGGT, located 112 bp and 57 bp upstream of the transcriptional start (Fig. 2A). Furthermore, two potential RamB binding sites were detected, located 132 bp upstream (TATTTTTCGAAAT; completely conserved nucleotides underlined) and 96 bp downstream (AGCAATTTGCCAA) of the *adhA* transcrip-

tional start site (Fig. 2A). These observations suggested a direct interaction of RamA and RamB with the *adhA* promoter region and prompted us to perform EMSAs with His-tagged RamA and RamB proteins and the $adhAp_1$, $adhAp_2$, and $adhAp_3$ fragments (Fig. 2B and C). The incubation of $adhAp_1$ and $adhAp_3$ with RamA resulted in a three-step retardation of the probe (Fig. 2B), probably due to the binding of RamA to the three postulated RamA binding sites. The incubation of RamA with $adhAp_2$ resulted in a one-step retardation of the probe, probably due to the binding of RamA to the single RamA binding site 57 bp upstream of the transcriptional start. The incubation of $adhAp_1$ with RamB led to a two-step retardation signal (Fig. 2C), whereas the incubation of RamB with $adhAp_2$ and $adhAp_3$ led to a one-step retardation of the respective probes. We also tested for an interaction of RamA and RamB during the binding to the $adhAp_1$, $adhAp_2$, and $adhAp_3$ fragments. However, neither combined incubation with RamA and RamB nor the prebinding of RamA or RamB blocked or enhanced the binding of the other regulator (data not shown). These results are in agreement with the postulated RamA and RamB binding sites and indicate a direct participation of RamA and of RamB in *adhA* gene expression control in *C. glutamicum*.

RamA and RamB control the *adhA* gene expression. To test for a regulatory function of RamA and RamB in *adhA* expression, the specific ADH activities were determined in the *ramB*-deficient mutant *C. glutamicum* RG1 and in the *ramA*-deficient mutant *C. glutamicum* RG2 (Table 2). When grown in minimal medium containing ethanol, *C. glutamicum* RG1 showed about the same specific ADH activity as WT *C. glutamicum*. When grown on glucose or ethanol plus glucose and, also, when grown on acetate or a mixture of ethanol and acetate, *C. glutamicum* RG1 showed significantly (two- to fivefold) higher specific ADH activities than WT *C. glutamicum*. In contrast to *C. glutamicum* RG1, the RamA-deficient strain RG2 was not able to grow on ethanol, on acetate, or on ethanol plus acetate and showed no ADH activity on the other substrates tested (Table 2).

To study the direct influence of RamA and RamB on *adhA* promoter activity, the specific CAT activities were determined in the respective mutant strains containing plasmids pET2-$adhAp_1$ and pET2-$adhAp_2$ (Table 3). The RamA-negative strains *C. glutamicum* RG2(pET2-$adhAp_1$) and *C. glutamicum* RG2(pET2-$adhAp_2$) showed no promoter activities at all. However, in *C. glutamicum* RG1(pET2-$adhAp_1$), we detected twofold higher specific CAT activities on glucose and on ethanol plus glucose and about threefold higher activities on acetate and ethanol plus acetate than in WT *C. glutamicum*-(pET2-$adhAp_1$). The specific CAT activities on ethanol were similar in both WT *C. glutamicum*(pET2-$adhAp_1$) and *C. glutamicum* RG1(pET2-$adhAp_1$) (Table 3). Interestingly, the specific CAT activities in *C. glutamicum* RG1(pET2-$adhAp_2$) were similar to the activities determined in WT *C. glutamicum*-(pET2-$adhAp_2$) on all substrates tested. Taken together, the results indicate an involvement of both RamA and RamB in the expression control of *adhA*. RamA is absolutely required for the expression of the *C. glutamicum adhA* gene and, thus, for ADH activity. RamB exerts a negative control when the cells grow in the presence of glucose or acetate as the sole or as an additional carbon source. However, the results also indicate that neither RamA nor RamB alone is responsible for the carbon source-dependent ADH regulation, i.e., for complete *adhA* repression in the presence of glucose or acetate in the growth medium. It is feasible that different RamA abundance within the cells (and thus, different *adhA* activation), together with RamB, controls *adhA* expression during growth on different substrates. Alternatively, *C. glutamicum* might possess an additional transcriptional regulator(s) controlling *adhA* expression and, together with RamB, being responsible for catabolite repression in medium containing ethanol and glucose or acetate.

DISCUSSION

C. glutamicum is able to grow on ethanol as the single source of carbon and energy, and it has been shown that the enzymes AK, PTA, ICL, and MS are essential for growth on this substrate (3). Here we identified and characterized the *C. glutamicum adhA* gene encoding an ADH which is essential for ethanol oxidation. ADH proteins are ubiquitous enzymes and widespread in all life forms, fulfilling quite different physiological functions. Some are involved in the formation and others in the degradation of alcohols. The best-studied ADHs are the long-chain, zinc-dependent enzymes of group I, represented by the ADHI of *S. cerevisiae* (reviewed in reference 41). Group II ADHs consist of short-chain, zinc-independent enzymes, and group III contains iron-activated ADHs, represented by the CoA-dependent bifunctional enzymes from *E. coli* (AdhE) and *Clostridium acetobutylicum* (16, 22). BLAST analysis showed high similarities of the *C. glutamicum adhA* gene product with group I ADHs, and sequence alignments revealed highly conserved residues and sequence motifs in those regions suggested to be essential for cofactor binding and catalytic activity. Among these were the GXGXXG pattern (position 180 to 185) in the coenzyme-binding fold and a highly conserved aspartate (Asp-203) (28, 34, 41, 46). This aspartate residue corresponds well with the finding that the *C. glutamicum* ADH activity was specific for NAD^+ (3). Furthermore, the GHEX XGXXXXXGXXV motif containing the second ligand of the catalytic zinc (i.e., His-69) (28, 41) is highly conserved (position 68 to 82). Taken together, all these data indicate that the ADH of *C. glutamicum* is a member of the group I ADHs.

The transcriptional characterization of the *C. glutamicum adhA* revealed a monocistronic organization and a carbon source-dependent expression. It was induced in the presence of ethanol and repressed by glucose. The repression of *adhA* was also observed when both carbon sources were present in the medium, in agreement with the low specific ADH activity detected on the substrate mixture. As shown previously, we also observed low ALDH, AK, PTA, ICL, and MS activities on ethanol-glucose (3), suggesting a repression of all the corresponding genes in the presence of both these carbon sources. The biphasic growth behavior observed on the ethanol-glucose mixture is thus obviously due to the repression of all the genes in the presence of glucose and derepression when glucose was exhausted.

Interestingly, *adhA* was also repressed in the presence of acetate. Acetate-mediated *adhA* repression is corroborated by DNA microarray experiments showing lower *adhA*-RNA levels in acetate-grown *C. glutamicum* cells than in glucose-grown

cells (37). Since the ADH and the ALDH reactions in principle are reversible, it can be speculated that the acetate-mediated repression of *adhA* prevents ethanol formation from acetate. Above all, we detected a biphasic growth behavior of *C. glutamicum* during growth on the ethanol-acetate substrate mixture, with acetate utilization preferred (data not shown). The favored acetate metabolization in the presence of ethanol was unexpected, since the results of Wendisch et al. (53) indicated a parallel substrate metabolization of acetate and other carbon sources, such as lactate, succinate, or glutamate. Furthermore, in contrast to the *adhA* gene, the *pta-ack* operon, *aceA*, and *aceB* showed high levels of expression in the presence of acetate (reviewed in reference 20). These results led us to conclude that the observed biphasic growth behavior on ethanol-acetate is due to the repression of the *adhA* gene in the presence of acetate and derepression after acetate consumption. However, the preferred utilization of acetate or other organic acids is not uncommon in other soil bacteria, as shown for, e.g., pseudomonads, *Azotobacter vinelandii*, *Acinetobacter baylyi* ADP1, or *Ralstonia eutropha* (2, 11, 13, 18).

As shown here, ethanol utilization is tightly regulated in *C. glutamicum*, and this has also been shown for other organisms. The genes encoding the components of the ethanol oxidation system of *P. aeruginosa* are positively regulated by AgmR. Furthermore, the two-component system ExaDE controls the expression of *exaA*, the gene for the pyrroloquinoline quinone-dependent ADH (21, 23). *ADH2*, the ethanol-oxidizing ADHII gene of *S. cerevisiae*, was repressed in the presence of glucose or other fermentative carbon sources and activated by Adr1 when glucose was exhausted (17, 54). In *Aspergillus nidulans*, the *alc* genes of the ethanol utilization pathway are also subject to two regulatory mechanisms: the positive transcriptional regulation mediated by AlcR and the negative control of CreA in the presence of more favorable growth substrates, such as glucose or acetate (reviewed in reference 15).

In contrast to the well-characterized regulation of ethanol metabolism in some other organisms, so far nothing was known on the regulation of ethanol utilization in *C. glutamicum*. However, the results obtained in this study show that the two regulators of acetate metabolism, RamA and RamB, are involved in *adhA* regulation. Similar to the regulation of the *pta-ack* operon, *aceA*, and *aceB* (10), RamA activates the *adhA* gene and, in fact, is essential for *adhA* expression on any carbon source tested. As previously shown for the control of the expression of the RamA gene itself (9), the level of *adhA* expression is dependent on the number of RamA binding sites in the promoter region. A dependence of the binding-site number on the level of gene expression has been also assumed for genes controlled by AmtR, the repressor of nitrogen control in *C. glutamicum*, and for the CreA-mediated regulation of *alcR* in *A. nidulans* (4, 15). In contrast to RamA, RamB was found to exert a negative control on *adhA* expression. Similar to the control of the AK, PTA, ICL, and MS genes (19), RamB represses *adhA* expression in the presence of glucose. RamB also represses *adhA* in the presence of acetate, and this is in contrast to the control of the former genes, which are activated in the presence of acetate. However, the *adhA* gene in the RamB-deficient mutant *C. glutamicum* RG1 was not completely derepressed in the presence of glucose or acetate. This finding might be due to a lower intracellular abundance of RamA and thus, in fact, to an incomplete activation of *adhA* expression under these conditions. Alternatively, RamB might not be the only regulator involved in the glucose- and acetate-dependent repression of *adhA*. Interestingly, although in vitro binding of RamB to both potential RamB binding sites was shown, only the motif 132 bp upstream of the transcriptional start site exhibited a physiological function under the conditions tested. All these results led us to suppose that the RamB-mediated repression of *adhA* is different from that of the *pta-ack* operon, *aceA*, and *aceB*. We propose the requirement of other effector molecules and, possibly, the involvement of other proteins in *adhA* repression. A candidate for involvement might be GlxR, a cAMP-dependent regulator (31), since we observed a sequence motif (TGTTG-N_6-ACACA) with similarity to the proposed consensus sequence of GlxR (TGT GA-N_6-ACACT) (35) in the *adhA* promoter region, centered 21 bp downstream of the *adhA* transcriptional initiation site. GlxR has been shown to be involved in the glucose-induced repression of the genes of the gluconate metabolism (*gntP* and *gntK*) (35), and due to the observations of potential binding sites, it has been supposed to be involved in the carbon source-dependent regulation of other genes, such as *acn* (encoding aconitase) or *gluA* (encoding a component of the glutamate uptake system) (35). However, further studies should focus on the involvement of GlxR in the regulation of *adhA*.

As outlined in the introduction, carbon catabolite repression is an important global regulatory system that has been investigated in several organisms (6, 45). In contrast, very little is known about the carbon catabolite repression mechanism in *C. glutamicum* and closely related *Corynebacterineae*, such as other corynebacteria or mycobacteria. The investigation of the regulation of ethanol metabolism in *C. glutamicum* and the identification of the participating regulators should help to elucidate catabolite repression in these organisms.

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